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# Genomic but Not Antigenomic Hepatitis Delta Virus RNA Is Preferentially Exported from the Nucleus Immediately after Synthesis and Processing

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**Hepatitis delta virus (HDV) contains a viroid-like circular RNA that replicates via a double rolling circle replication mechanism. It is generally assumed that HDV RNA is synthesized and remains exclusively in the nucleus until being exported to the cytoplasm for virion assembly. Using a [$^{32}$P]orthophosphate metabolic labeling procedure to study HDV RNA replication (T. B. Macnaughton, S. T. Shi, L. E. Modahl, and M. M. C. Lai. J. Virol. 76:3920–3927, 2002), we unexpectedly found that a significant amount of newly synthesized HDV RNA was detected in the cytoplasm. Surprisingly, Northern blot analysis revealed that the genomic-sense HDV RNA is present almost equally in both the nucleus and cytoplasm, whereas antigenomic HDV RNA was mostly retained in the nucleus, suggesting the specific and highly selective export of genomic HDV RNA. Kinetic studies showed that genomic HDV RNA was exported soon after synthesis. However, only the monomer and, to a lesser extent, the dimer HDV RNAs were exported to the cytoplasm; very little higher-molecular-weight HDV RNA species were detected in the cytoplasm. These results suggest that the cleavage and processing of HDV RNA may facilitate RNA export. The export of genomic HDV RNA was resistant to leptomycin B, indicating that a cell region maintenance 1 (Crm1)-independent pathway was involved. The large form of hepatitis delta antigen (L-HDAg), which is responsible for virus packaging, was not required for RNA export, as a mutant HDV RNA genome unable to synthesize L-HDAg was still exported. The proportions of genomic HDV RNA in the nucleus and cytoplasm remained relatively constant throughout replication, indicating that export of genomic HDV RNA occurred continuously. In contrast, while antigenomic HDV RNA was predominately in the nucleus, there was a proportionally large fraction of antigenomic HDV RNA in the cytoplasm at early time points of RNA replication. These findings uncover a previously unrecognized presence of HDV RNA in the cytoplasm, which may have implications for viral RNA synthesis and packaging.**

Hepatitis delta virus (HDV) is a small RNA virus that is associated with severe acute and chronic liver disease in humans. HDV has been classified as a subviral satellite of hepatitis B virus (HBV) due to an obligate relationship with HBV infections in nature. However, unlike other satellite viruses, which require the helper virus for genome replication, HDV depends on HBV only for an envelope of hepatitis B surface antigen (HBsAg) (reviewed in reference 18). The HDV genome consists of a small, single-stranded, circular RNA molecule of roughly 1.7 kb that, in the virion, is associated in a ribonucleoprotein complex with the only known virus-encoded protein, hepatitis delta antigen (HDAg) (24). HDV RNA also contains a ribozyme activity, which is essential for HDV RNA replication (8, 14). The genomic form of HDV RNA does not encode any protein. However, the complementary strand (antigenomic HDV RNA), which is detected in HDV-infected cells, encodes HDAg. HDAg occurs as two species, small HDAg (S-HDAg or p24) and large HDAg (L-HDAg or p27), which play different roles in HDV replication. S-HDAg is an essential activator of HDV RNA replication (9), whereas L-HDAg has a potent inhibitory activity (5) but is essential for virion assembly (2). L-HDAg is synthesized only late in the viral replication cycle, as a result of an RNA editing event, which alters the termination codon of the open reading frame for S-HDAg (12). While the mechanism that enables these two HDAg species to exert their different modulatory effects on HDV replication is not understood, these activities are dependent on the ability of the two proteins to undergo protein-protein binding reactions (28). Both S- and L-HDAg are localized almost exclusively in the nucleus (23), although some redistribution of these proteins within the nucleus during HDV RNA replication has been noted (13, 27). HDV RNA has also been reported to reside primarily in the nuclei of HDV-infected hepatocytes (6). However, virus assembly presumably takes place in the cytoplasm, where HBsAg is located. Thus, it is generally assumed that HDV RNA is replicated exclusively in the nucleus and is retained in the nucleus until the time when virus assembly is ready to occur, probably after L-HDAg is synthesized. Despite the obvious requirement for HDV RNA to be exported at certain points in the viral life cycle to the cytoplasm, where the HDV particle is assembled, the possible presence of HDV RNA in the cytoplasm has so far not been addressed experimentally.

Recent studies indicate that there are fundamental differences between the mechanisms of HDV genomic and antigenomic RNA synthesis. For example, L-HDAg potently inhibits genomic but not antigenomic HDV RNA synthesis (19); re-

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combinant HDAg can initiate HDV RNA synthesis only from genomic, not antigenomic, HDV RNA (25); genomic, not antigenomic, HDV RNA synthesis requires phosphorylation of amino acid 177 of S-HDAg (21). Furthermore, some differences between the intranuclear distributions of genomic and antigenomic HDV RNA were observed; specifically, antigenomic HDV RNA was localized near nuclear domain 10, whereas genomic HDV RNA had a more uniform distribution throughout the nucleus (1). Moreover, in an accompanying study (15), we show that, in lysolecithin-permeabilized cells, synthesis of genomic HDV RNA was highly sensitive to α-amanitin, suggesting the involvement of host cell RNA polymerase II (pol II). In contrast, synthesis of antigenomic HDV RNA was completely resistant, suggesting that a different host cell transcription machinery is involved in this process (15, 20).

In this study, we have identified a further feature distinguishing between genomic and antigenomic HDV RNA synthesis and in so doing discovered a previously unrecognized export pathway in HDV-replicating cells. The unexpected presence of HDV RNA in the cytoplasm may have implications for viral RNA synthesis as well as viral packaging.

## MATERIALS AND METHODS

**Cell culture and transfection.** The human hepatoma cell line HuH7 (22) was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin and was incubated at 37°C in 5% $CO_2$. For transfection studies, cell cultures were seeded overnight in six-well plates or 60-mm-diameter petri dishes and transfected with 5 and 10 μg, respectively, of 50:50 mixtures of HDAg mRNA and HDV RNA (1.2 times the genome-length) using DMRIE-C reagent (Gibco BRL) according to the manufacturer's directions (15). Following transfection, the cultures were incubated overnight, the medium was changed, and incubation was continued for up to an additional 7 days.

**Plasmids and cloning.** Plasmids pBSδ1.2G and pBSδ1.2AG (in vitro transcription templates for the genomic and antigenomic HDV RNAs, respectively, which were 1.2 times the genome length) were described in the accompanying study (15). The mutant HDV sequence containing tandem in-frame stop codons was prepared with a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's directions. The reaction was performed with plasmid pCδ1.2G as a template and complementary primers 5′-AACCAGGGATTTCC ATAGTGATATACTCTTCCCAGCC-3′ and 5′-GGCTGGGAAGAGTATAT CACTATGGAAATCCCTGGTT-3′ (the inserted nucleotides are underlined). pCδ1.2G contains the same HDV cDNA insert as pBSδ1.2G cloned between the *Eco*RV and *Xba*I sites of plasmid pCDNA3 (Clontech). In vitro transcription templates for genomic and antigenomic HDV(2xS) RNA [pBSδ1.2G(2xS) and pBSδ1.2AG(2xS), respectively] were prepared by cloning *Xba*I monomer fragments of mutant HDV cDNA from pCδ1.2G into the *Xba*I sites of pBSδG-Basic and pBSδAG-Basic (15), respectively. Plasmid pX9-1/II, used as an in vitro transcription template for HDAg mRNA synthesis, and pTMδSalA and pTMδSalB, used for the generation of unlabeled and $^{32}$P-labeled monomers of genomic and antigenomic HDV RNA, respectively, have been described elsewhere (13, 17). pBSδHX, used in the generation of $^{32}$P-labeled riboprobes for detection of the antigenomic HDV RNA outside the HDAg mRNA sequence, was described in the accompanying study (15).

**In vitro transcription.** HDV RNAs 1.2 times the genome length were transcribed from plasmids pBSδ1.2G, pBSδ1.2AG, pBSδ1.2G(2xS), and pBSδ1.2AG (2xS) with T7 MEGAscript kits (Ambion) after linearization with restriction enzyme *Not*I. Capped mRNA for HDAg was transcribed from plasmid pX9-I/II after linearization with *Hin*dIII by using a T7 m-Message m-Machine kit (Ambion). Unlabeled monomer genomic and antigenomic HDV RNAs were transcribed from pTMδSalA and pTMδSalB with T7 MEGAscript after linearization by *Pst*I digestion. The method for generation of HDV-specific $^{32}$P-labeled riboprobes has been described elsewhere (13).

**Partitioning of nuclear and cytoplasmic fractions.** Partitioning of cell lysates into nuclear and cytoplasmic fractions was performed by a modification of a previously published protocol (16). The entire procedure was performed on ice with prechilled reagents. Briefly, 60-mm-diameter petri dish cultures were washed once with phosphate-buffered saline (PBS); the cells were then scraped into 1 ml of PBS, pelleted by centrifugation (20 s, approximately 3,000 × *g*), and resuspended in 200 μl of lysis mixture (100 mM NaCl, 10 mM Tris-HCl [pH 7.4], 1 mM EDTA,1% NP-40 [Roche Molecular Biochemicals]). After incubation for 4 min, the tube was centrifuged briefly, as described above, and the supernatant, which represents the cytoplasmic fraction, was transferred to a fresh microcentrifuge tube. The pellet, which represents the nuclear fraction, was then washed once with 1 ml of lysis buffer to minimize contamination with the cytoplasmic fraction prior to further processing.

**Northern blot and reverse hybridization analysis.** RNA was extracted from intact cells or nuclear and cytoplasmic fractions using Tri-Reagent (Molecular Research Center, Inc.) according to the manufacturer's protocol. For analysis by Northern blotting, the RNA samples were treated with formaldehyde and separated by electrophoresis through MOPS (morpholinepropanesulfonic acid)-formaldehyde-containing 1.2 to 1.8% agarose gels. RNA was then transferred to a BrightStar-Plus nylon membrane (Ambion) according to the method recommended by the manufacturer. Hybridizations for the detection of genomic and antigenomic HDV RNA were performed at 68°C with ULTRAhyb reagent (Ambion) and in vitro-transcribed $^{32}$P-labeled probes generated from pTMδSalB and pBSδHX, respectively. The membrane was washed at 75°C and exposed to Biomax MR or MS X-ray films (Kodak). Quantitation was performed by phosphorimagery using ImageQuant, version 1.11, software (Molecular Dynamics). Detection of ChoA mRNA (7) was performed as described previously (20).

For analysis by reverse hybridization, probes were fixed to the membrane by applying 1 μl (1 μg) of heat-denatured, unlabeled RNA samples directly to strips of a BrightStar Plus membrane and immobilizing them by baking at 80°C for 30 min in a vacuum oven. The probes used were as follows. For the detection of genomic and antigenomic HDV RNA, unlabeled in vitro-transcribed RNA was derived from pTMdSalB and pTMdSalA, respectively, with T7 RNA polymerase. Hybridizations and stringent washes for the detection of HDV RNA were performed as described above.

**[$^{32}$P]orthophosphate metabolic labeling.** Metabolic labeling using [$^{32}$P]orthophosphate was performed at either 37 or 40°C in the presence of 50 μg of actinomycin D (Fisher Biotech)/ml as described in the accompanying study (15). For analysis of the effect of leptomycin B (Sigma) on nucleocytoplasmic transport, this inhibitor was added to a final concentration of 3 ng/ml for 2 h prior to and throughout the [$^{32}$P]orthophosphate labeling period.

## RESULTS

**Genomic HDV RNA is preferentially exported to the cytoplasm.** Since HDAg is detected mainly in the nuclei of HDV-infected hepatocytes (23), it is generally assumed that this is the site of HDV RNA synthesis. In situ hybridization studies have shown that most of the HDV RNA is localized in the nucleus of HDV-infected hepatocytes; however, some HDV RNA was detected in the cytoplasm (6). While the latter could represent RNA for viral particle assembly, the possibility that the cytoplasm was also a site of HDV RNA replication could not be strictly ruled out. In an accompanying study we succeeded in detecting intermediates of HDV RNA replication by in vivo labeling of HDV RNA with [$^{32}$P]orthophosphate (15). We unexpectedly found, in a nuclear and cytoplasmic partitioning experiment designed to enrich the HDV RNA-specific fraction, that a significant amount of $^{32}$P-labeled HDV RNA was present in the cytoplasm.

To further investigate this unexpected observation, HuH7 cells were labeled for 4 h with [$^{32}$P]orthophosphate in the presence of actinomycin D 3 or 4 days after transfection with HDV RNA and HDAg mRNA. The cells were then partitioned into nuclear and cytoplasmic fractions, and the RNA was extracted and separated on 1.2% denaturing agarose gels (Fig. 1). The results showed that RNA from HDV RNA-transfected cells contained at least two specific bands in both the nucleus and cytoplasm which were not in the mock-transfected cells (Fig. 1, compare lanes 2 and 3 to lanes 4 to 7). As

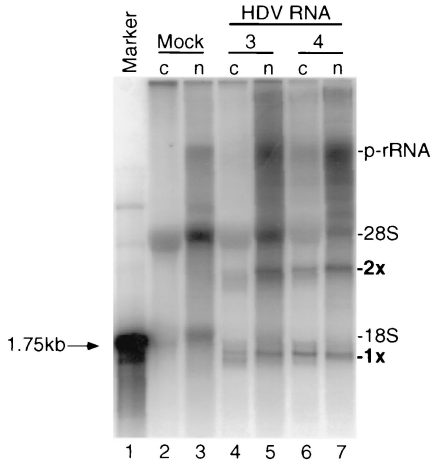


FIG. 1. Newly synthesized HDV RNA is present in both nuclear and cytoplasmic fractions. $^{32}$P-labeled RNAs from HDV RNA-transfected (lanes 4 to 7) and mock-transfected (lanes 2 and 3) HuH7 cells were separated into nuclear (n) and cytoplasmic (c) fractions. RNA was separated by electrophoresis on a 1.2% denaturing agarose gel. Lane 1, 1.75-kb RNA marker. 1x and 2x, positions of monomer and dimer HDV RNAs, respectively; p-rRNA, 28S, and 18S, cellular rRNA precursor and 28S and 18S rRNA species, respectively. RNA labeling was performed on either day 3 (lanes 4 and 5) or 4 (lanes 6 and 7) posttransfection.

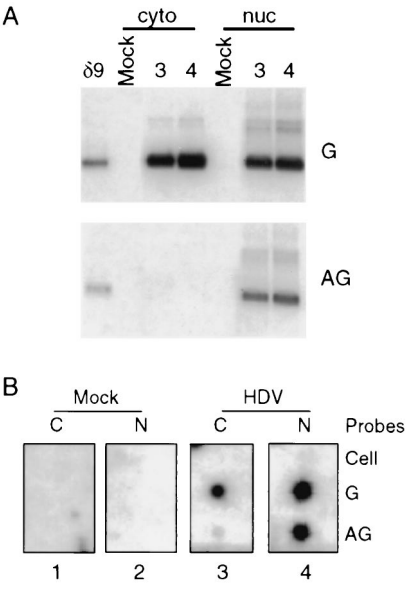


FIG. 2. Genomic HDV RNA is preferentially exported to the cytoplasm. (A) Northern blot analysis to detect genomic (G) and antigenomic (AG) HDV RNA from nuclear (nuc) and cytoplasmic (cyto) fractions of HuH7 cells at days 3 and 4 posttransfection. Lane δ9, genomic HDV RNA marker (13). (B) Reverse hybridization analysis of $^{32}$P-labeled nuclear (N) and cytoplasmic (C) RNA from mock- and HDV RNA-transfected HuH7 cells at 4 days posttransfection. Probes were specific for genomic and antigenomic HDV RNA. Cell, probe consisting of the total cellular RNA extracted from untransfected cells.

described in the accompanying study (15), these two bands represent monomeric and dimeric HDV RNA. Surprisingly, both monomer and dimer HDV RNAs were almost equally distributed between nuclear and cytoplasmic fractions at both day 3 and day 4 posttransfection. This result suggested that either HDV RNA synthesis occurs in both these subcellular compartments or HDV RNA is synthesized in the nucleus and rapidly transported to the cytoplasm or vice versa. The efficient separation of the nuclear and cytoplasmic fractions was evidenced by the finding that the cellular precursor rRNA (Fig. 1) was largely restricted to the nuclear fractions whereas the 28S and 18S rRNA species were more abundant in the cytoplasmic fractions. Other cellular and viral RNAs also were well separated between these two subcellular fractions (see below), indicating very little cross-contamination between the nucleus and cytoplasm in our preparations.

To investigate these issues further, we subjected duplicate, unlabeled RNA samples to Northern blot hybridization to detect genomic and antigenomic HDV RNA (Fig. 2A). Genomic monomeric HDV RNA was present in almost equal amounts in the cytoplasmic and nuclear fractions. However, there was relatively more dimeric and trimeric HDV RNA in the nucleus than in the cytoplasm. In contrast, antigenomic HDV RNA was detected almost exclusively in the nuclear fractions (Fig. 2A), confirming that the detection of genomic HDV RNA in the cytoplasm was not due to contamination with nuclear RNA. Longer exposure of the blot revealed a low level of monomer antigenomic HDV RNA in the cytoplasm (data not shown); whether this is the result of a specific event or a low-level nuclear contamination remains to be resolved.

To ascertain the polarity of $^{32}$P-labeled HDV RNA in the cytoplasm, we subjected labeled nuclear and cytoplasmic RNA to reverse hybridization analysis as described previously (15). The results showed that, while genomic HDV RNA was present in both the nucleus and cytoplasm, the antigenomic HDV RNA was mostly in the nucleus (Fig. 2B, compare panels 3 and 4). None of the samples hybridized to the total cellular RNA probes, nor did the RNA extracted from mock-transfected cells hybridize with any of the HDV-specific probes. Thus, both newly synthesized and steady-state genomic HDV RNAs were present in both the nucleus and cytoplasm, whereas the antigenomic HDV RNAs were present almost exclusively in the nucleus. Since antigenomic HDV RNA is the template for genomic HDV RNA synthesis, the finding that antigenomic species were localized almost entirely in the nucleus suggests that HDV RNA synthesis takes place in the nucleus. Taken together, these results indicate that genomic HDV RNA is continually and preferentially exported from the nucleus while antigenomic HDV RNA is retained almost exclusively in the nucleus.

**HDV RNA export from the nucleus to the cytoplasm occurs rapidly after synthesis.** To further examine the relationship between the nuclear and cytoplasmic HDV RNA species, we studied the kinetics of appearance of HDV RNA in these two subcellular compartments. For this, [$^{32}$P]orthophosphate metabolic labeling was performed at 40°C for from 30 to 240 min and the labeled cells were partitioned into nuclear and cytoplasmic fractions (Fig. 3A). The higher incubation temperature increases the rate of HDV RNA synthesis (15), allowing easier detection of HDV RNA in samples labeled for shorter time periods. The same quantity of the $^{32}$P label was loaded into each lane; this resulted in a requirement for almost 10 times more RNA from cultures labeled for 30 min than from cells

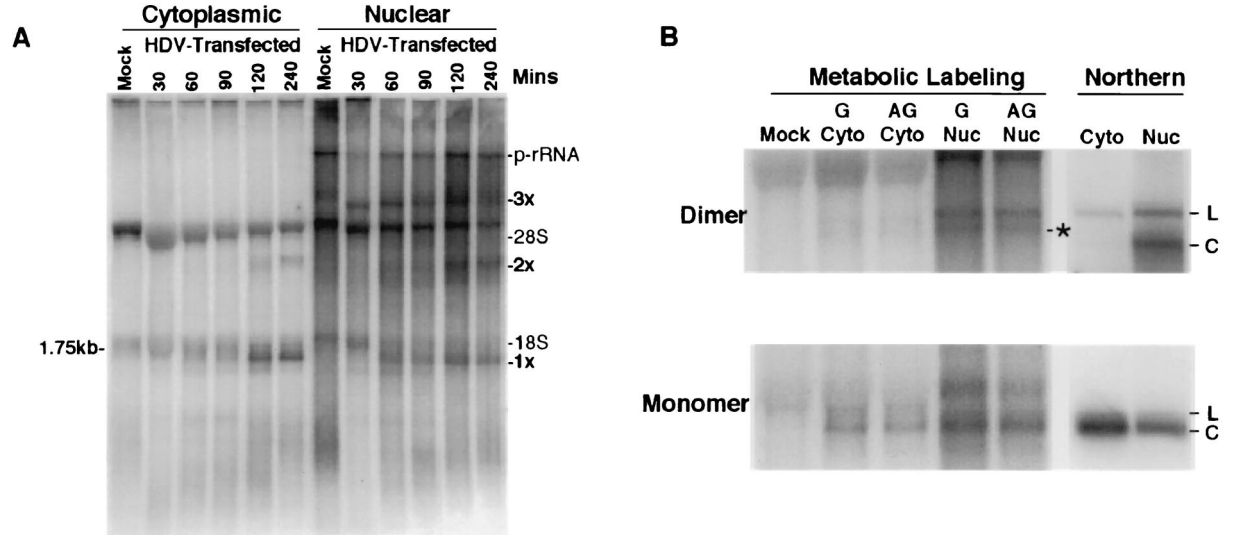


FIG. 3. Nuclear export of HDV RNA occurs rapidly after synthesis and favors the highly processed species. (A) Nuclear and cytoplasmic RNA from HDV-transfected cells following 30 to 240 min of [$^{32}$P]orthophosphate metabolic labeling at 40°C. The length of labeling is shown at the top of each track. Mock-transfected HuH7 cells were labeled for 240 min. Monomer (1x), dimer (2x), and trimer (3x) HDV RNA species are marked. p-rRNA, 28S and 18S, rRNA species as described in the legend to Fig. 1. RNA was separated on 1.2% denaturing agarose gel. (B) Separation of linear and circular HDV RNA species. Nuclear (Nuc) and cytoplasmic (Cyto) RNA from unlabeled HuH7 cells transfected with antigenomic HDV RNA (Northern) or from $^{32}$P-labeled cells transfected with either genomic (G) or antigenomic (AG) HDV RNA were separated on a 1.8% denaturing agarose gel. The unlabeled RNA was analyzed by Northern blotting to detect genomic HDV RNA, while the $^{32}$P-labeled RNA was detected by autoradiography. L and C, linear and circular forms, respectively; asterisk, novel HDV RNA transcript in $^{32}$P-labeled cells.

labeled for 240 min and also resulted in a slight mobility shift between the same RNA species from the nucleus and from the cytoplasm. Both monomeric and dimeric HDV RNAs were detected in the nuclear fraction after 60 min of labeling; the bands became slightly stronger as the labeling period increased. Several even slower migrating bands consistent in size with trimeric and longer multimeric species of HDV RNA were detected in the nuclear fraction following 240 min of labeling. In contrast, only monomeric and dimeric HDV RNA species were detectable in the cytoplasm. The monomer appeared in the cytoplasm first (very faintly after 60 min of labeling). The dimer was not detected until 120 min of labeling. The bands for both species became stronger with longer incubation times. The time lapses between the appearance of these two HDV RNA species in the nucleus and in the cytoplasm are most consistent with the interpretation that HDV RNA was synthesized in the nucleus and then exported to the cytoplasm. Moreover, the fact that the dimeric RNA was not detected in the cytoplasm until 60 min after detection of the monomer RNA in the cytoplasm suggests that the smaller and more extensively processed HDV RNA species are exported more rapidly. Consistent with this interpretation, the ratios of dimeric to monomeric HDV RNA after 240 min of labeling were approximately 1:1 in the nucleus and 1:3 in the cytoplasm (Fig. 3A). Similarly, the proportions of dimeric and trimeric to monomeric HDV RNA in the nuclear fraction were higher than those in the cytoplasmic fraction in the Northern blot hybridization presented in Fig. 2A. As in Fig. 1, the rRNA precursor was almost entirely restricted to the nuclear fractions (Fig. 3A), confirming the clean separation of the nuclear and cytoplasmic samples.

We next examined the state (linear or circular) of the nuclear and cytoplasmic monomeric and dimeric HDV RNA species (Fig. 3B). For this purpose, $^{32}$P-labeled RNAs from nuclear and cytoplasmic fractions were separated with denaturing 1.8% agarose gels. Under the gel conditions used, circular HDV RNA runs with a slightly greater mobility than the linear forms (20). As a comparison, unlabeled nuclear and cytoplasmic RNAs from HDV RNA-transfected HuH7 cells were run on the same gel and analyzed by Northern blot hybridization (Fig. 3B). The $^{32}$P-labeled monomeric HDV RNA in both the nucleus and cytoplasm appeared to be mostly circular (Fig. 3B, bottom). This was similar to the Northern blot results, indicating that the monomer HDV RNA was rapidly circularized after synthesis. In contrast, most of the $^{32}$P-labeled dimeric HDV RNA in both the nucleus and cytoplasm appeared to be linear (Fig. 3B, top). The dimer HDV RNA detected by Northern blotting in the cytoplasm was mostly linear but included circular species in the nucleus. Thus, in contrast to the monomer, dimeric HDV RNA appears to circularize very inefficiently. The circularized dimer RNA was not exported to the cytoplasm.

Unexpectedly, an additional HDV-specific species with a mobility between those of the linear and circular dimeric forms was also detected in the metabolically labeled samples (Fig. 3B, top). We are currently unsure of the nature of this species, but circumstantial evidence suggests that it represents a linear form ca. 200 nucleotides smaller than the linear dimer.

**Nuclear export of genomic HDV RNA is independent of L-HDAg.** The nuclear export of genomic HDV RNA may be related to the virion assembly process and may not be part of HDV RNA replication per se. If this is so, it might be expected that its export would be dependent on the presence of L-HDAg, as suggested by a recent study (11). To address this

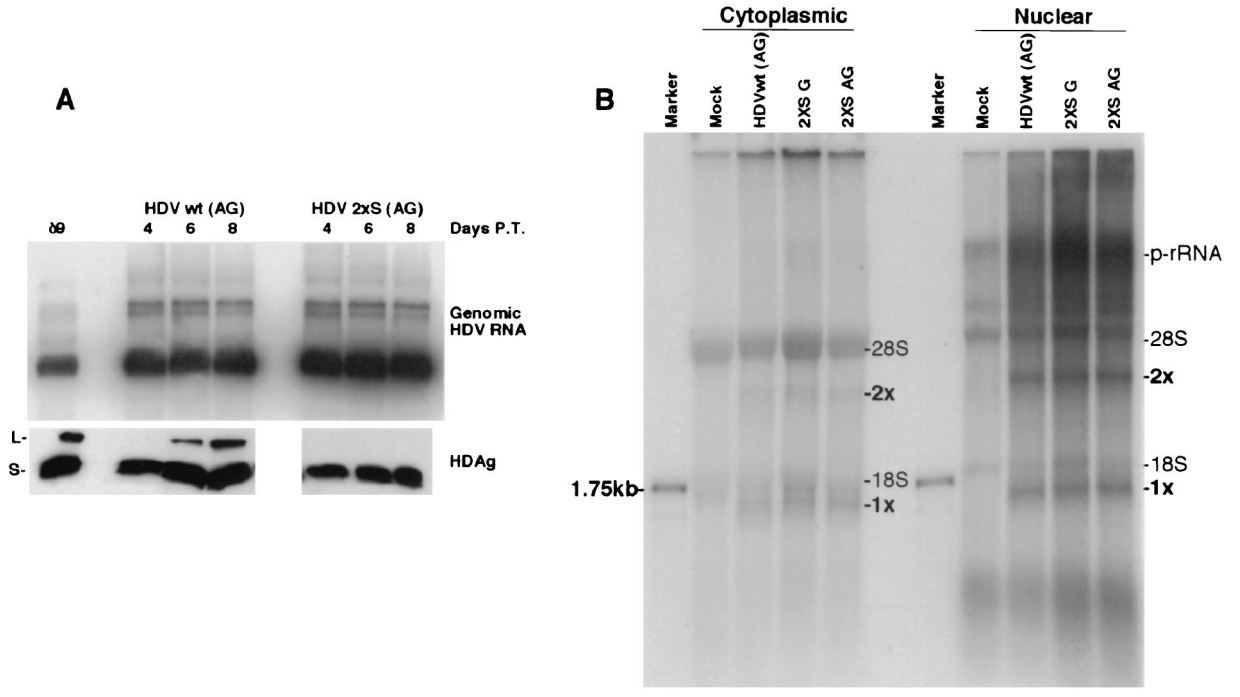


FIG. 4. HDV RNA export does not require L-HDAg. (A) (Top) Northern blot analysis to detect genomic HDV RNA from cells transfected with either wild type (wt) or mutant (2xS) antigenomic (AG) HDV RNA. RNA samples were harvested at days 4, 6, and 8 posttransfection. Lane δ9, genomic HDV RNA marker. (Bottom) Western blot of L-HDAg (L) and S-HDAg (S) from cells described above. (B) $^{32}$P-labeled nuclear and cytoplasmic RNA from cells transfected with either wild-type antigenomic or mutant genomic (2xS G) or antigenomic (2xS AG) HDV RNA. p-rRNA, 28S and 18S, rRNA species as described in the legend to Fig. 1.

question, we constructed a mutant HDV genome similar to that described in an earlier study (2), in which a uridine nucleotide was inserted immediately after the UAG stop codon for S-HDAg. The effect of this mutation was the creation of two sequential, in-frame stop codons (viz., UAG UGA). Thus, it was predicted that this mutant genome, designated HDV (2xS), would produce only S-HDAg even after the RNA editing event that normally leads to the production of L-HDAg (12). To examine the phenotype of this mutant, in vitro-transcribed antigenomic HDV RNA 1.2 times the genome length containing this mutation [HDV-AG(2XS)] was transfected together with HDAg mRNA into HuH7 cells. Cultures were then assayed for genomic HDV RNA by Northern blotting and for HDAg by Western blotting 4 to 8 days later (Fig. 4A). The mutant HDV RNA replicated with an efficiency similar to that of the wild-type genome at all time points (Fig. 4A, top). However, while L-HDAg gradually became more abundant in cells transfected with the wild-type HDV RNA (Fig. 4A, bottom), only S-HDAg was detected in cells transfected with HDV-AG(2xS), indicating that the mutant HDV genome was unable to synthesize L-HDAg. We next examined metabolically labeled HDV-transfected cells (Fig. 4B). Despite the absence of L-HDAg, there was no obvious difference in the level of HDV RNA species exported to the cytoplasm between cells transfected with mutant and wild-type HDV RNA. This result was confirmed by Northern blot hybridization (data not shown; see Fig. 6A). These data show unequivocally that L-HDAg is not responsible for HDV RNA export to the cytoplasm.

**HDV RNA export occurs by a Crm1-independent pathway.** The vast majority of eukaryotic cellular RNAs synthesized in the nucleus are exported to the cytoplasm. This process occurs at the nuclear pore complex and is mediated by various nucleocytoplasmic transport factors (reviewed in reference 4). One of the better characterized of these factors is referred to as cell region maintenance 1 (Crm1), which mediates the nuclear export of most of the small nuclear ribonucleoprotein particles as well as some viral RNAs including the Rev response element of human immunodeficiency virus factors (reviewed in reference 4). We examined whether Crm1 mediates the nuclear export of HDV RNAs by studying the distribution of metabolically labeled HDV RNA in the presence of Crm1-specific inhibitor leptomycin B (Fig. 5). This treatment had no effect on the proportions of HDV RNA in nuclear and cytoplasmic fractions, indicating that HDV RNA export occurs by a Crm1-independent pathway. This result was confirmed by Northern blot hybridization (data not shown).

**A proportionally large fraction of antigenomic HDV RNA is exported to the cytoplasm early in HDV RNA replication.** Finally we examined if the fraction of HDV RNA exported to the cytoplasm remains constant or changes with time during RNA replication. For this experiment, we used HDV-G(2xS) RNA, which does not synthesize L-HDAg, to avoid the possible complication from the effect this protein may have on RNA export. Consistent with earlier results with wild-type HDV RNA (Fig. 2A), genomic HDV RNA was present in nearly equimolar amounts in both the nucleus and cytoplasm at all time points between days 4 and 8 posttransfection (Fig. 6A) indicating that export of genomic HDV RNA occurred con-

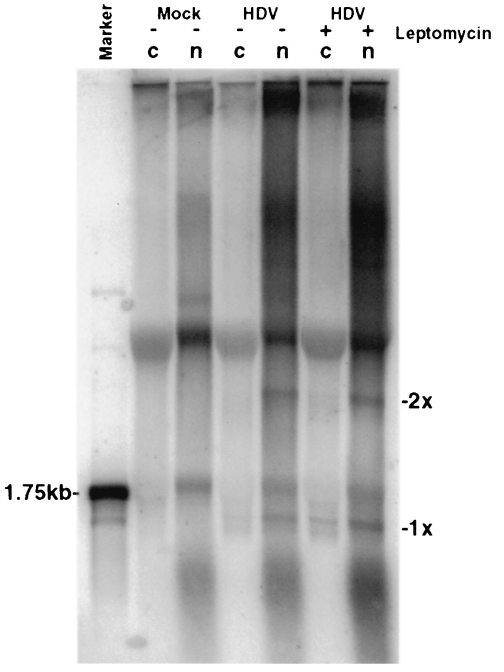


FIG. 5. HDV RNA export occurs by a Crm1-independent pathway. Shown is $^{32}$P-labeled RNA from nuclear (n) and cytoplasmic (c) fractions of transfected cells metabolically labeled in the presence (+) or absence (−) of 3 ng of leptomycin B/ml for 4 h. Labeling was performed 4 days posttransfection. 1x and 2x are as defined for Fig. 1.

tinuously. In contrast, antigenomic HDV RNA was almost exclusively in the nucleus, with only a trace amount being detected in the cytoplasm (Fig. 6A). A small amount of antigenomic HDV RNA was detected in the cytoplasm at days 7 and 8, probably due to nucleus-cytoplasm contamination due to cell death. Consistent with this interpretation, a small amount of cellular ChoA mRNA was detected in the nuclear fractions at the later time points. We next examined the subcellular distribution of HDV RNA at earlier time points (Fig. 6B). While the presence of input HDV RNA makes interpretation of genomic HDV RNA difficult at the very early time points, monomeric genomic HDV RNA was detected in both nuclear and cytoplasmic fractions by 42 h posttransfection. While some of this may represent the processing product of input RNA, it is likely that most was derived from subsequent HDV RNA replication, as the timing of the appearance of these genomic monomers coincided with the detection of significant levels of antigenomic HDV RNA (Fig. 6B). The ratio of genomic RNA in the nucleus to that in the cytoplasm was relatively constant over all time points. Taken together, these results indicate that export of genomic HDV RNA occurs continuously. In contrast, the distribution of antigenomic HDV RNA in nuclear and cytoplasmic fractions varied significantly with the time after transfection. While virtually no antigenomic HDV RNA was detected in cytoplasm 4 to 6 days after transfection (Fig. 6A), the nuclear and cytoplasmic antigenomic HDV RNA was nearly equimolar at 20 to 28 h posttransfection (Fig. 6B and C). The RNA became increasingly nuclear at later time points, such that, by 74 h posttransfection or later, the antigenomic HDV RNA was almost exclusively nuclear (compare Fig. 6A and B). These results could not be explained by the possible cross-contamination of the nuclear and cytoplasmic fractions, as the proportions of cellular ChoA mRNA for these fractions remained constant throughout the experiment (Fig. 6B). Thus, a substantial fraction of antigenomic HDV RNA is exported from the nucleus at the beginning of viral RNA replication. However, as RNA replication proceeds, this pathway is gradually shut down.

## DISCUSSION

In this study, we have identified a previously unrecognized HDV RNA export pathway during HDV RNA replication. Specifically, genomic HDV RNA was shown to be preferentially and rapidly exported to the cytoplasm soon after synthesis in the nucleus. Furthermore, a substantial amount of genomic RNA remains in the cytoplasm throughout the viral replication cycle. This discovery was a surprise, as it had always been thought that HDV RNA was localized primarily in the nucleus until it was ready to be packaged together with L-HDAg into virus particles. Our finding that L-HDAg is not required for the export of genomic HDV RNA suggests that this export event may be related to the process of RNA replication, in addition to its obvious role in virus assembly later in the viral life cycle. Consistent with this interpretation, the export of genomic HDV RNA was found to occur as soon as HDV RNA replication could be detected and continued for as long as the cells were in culture. Even the antigenomic HDV RNA is transported to the cytoplasm at the beginning of the RNA replication cycle. Since some of the cytoplasmic HDV RNA may be imported back to the nucleus through binding to HDAg (3), it is tempting to suggest that some steps of HDV RNA replication (e.g., RNA ligation and HDAg-RNA complex formation) may take place in the cytoplasm, at least during the early stages of the HDV replication cycle.

Several pieces of evidence indicate that the detection of HDV genomic RNA in the cytoplasm was not due to contamination of the cytoplasmic fraction by the nuclear fraction in our preparations. (i) While 28S and 18S rRNAs were detected in both the cytoplasmic and nuclear fractions, the precursor rRNA was detected almost exclusively in the nuclear fraction (Fig. 3A and 4). In contrast, ChoA mRNA was present exclusively in the cytoplasmic fraction (Fig. 6). There was a small amount of ChoA mRNA in the nuclear fraction on days 6 to 8 posttransfection, when the cells were very sick, indicating a slight cross-contamination between the nucleus and cytoplasm. However, in most of the experiments described in this study, the nucleus-cytoplasm separation was done early in the viral replication cycle. (ii) Only the genomic, not the antigenomic, HDV RNA was detected in the cytoplasmic fraction. Furthermore, the trimeric HDV RNA was detected only in the nucleus (Fig. 2 and 3A). The circular dimer RNA was detected only in the nucleus, not in the cytoplasm (Fig. 3B). Thus, there was clearly a selective and differential separation of the various HDV RNA species between the cytoplasm and nucleus, which could not be explained by the random leakage of nuclear RNA into the cytoplasm. (iii) In radiolabeling kinetics studies, the HDV monomer and dimer RNA species were detected first in the nucleus, approximately 30 to 60 min before they were detected in the cytoplasm (Fig. 3A). Also, at early time points after transfection (before 74 h posttransfection), proportion-

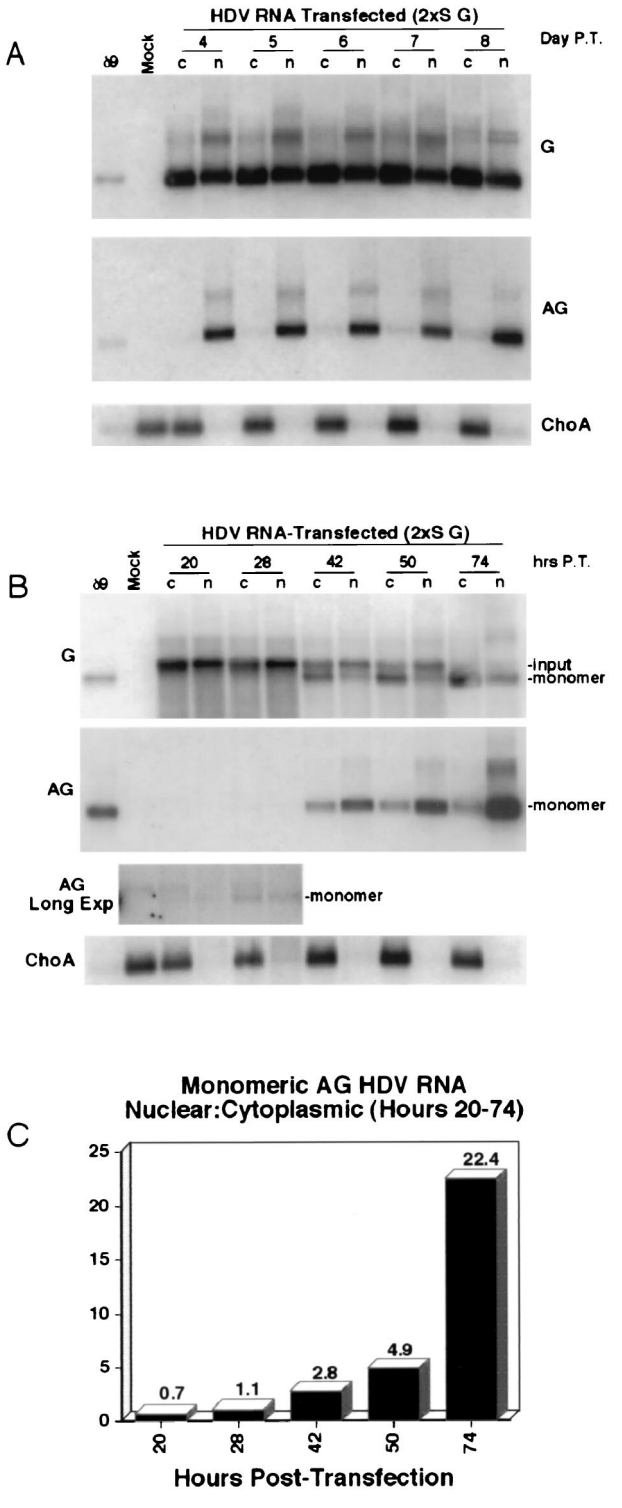


FIG. 6. Kinetic analysis of the appearance of genomic and antigenomic HDV RNA in nuclear and cytoplasmic fractions. (A) Northern blot analysis of genomic (G) and antigenomic (AG) HDV RNA in nuclear (n) and cytoplasmic (c) fractions, from 4 to 8 days posttransfection (P.T.), of HuH-7 cells transfected with mutant genomic (2xS G) HDV RNA. The ChoA blot shows detection of cellular ChoA mRNA in the same fractions. (B) Northern blot analysis of genomic and antigenomic HDV RNA in nuclear and cytoplasmic fractions from 20 to 74 h after transfection of HuH-7 cells with mutant genomic (2xS G) HDV RNA. Also indicated are HDV RNA species representing transfected (input) and unit length (monomer) HDV RNA. The ChoA blot shows detection of cellular ChoA mRNA in the same fractions. Autoradiographic exposure times for the AG and AG Long Exp (long exposure) blots were approximately 10 and 80 times, respectively, that for the G blot. (C) Quantitation for antigenomic HDV RNA from panel B. Data are presented as the ratios of monomeric RNA in the nuclear fractions to those in the cytoplasmic fractions.

ally larger portions of the HDV antigenomic RNA were present in the cytoplasm than at later time points. The cytoplasmic antigenomic RNA proportion decreased with time in a temporally regulated manner (Fig. 6C). Again, the temporally regulated appearance of these HDV RNA species in the cytoplasm could not be explained by the possible cross-contamination between the nuclear and cytoplasmic fractions or random leakage of the nucleus. (iv) The cytoplasmic HDV genomic RNA was detected both by metabolic labeling (in the presence of actinomycin D) and by Northern blotting (without actinomycin D). Thus, the possible actinomycin D toxicity could not explain these results. (v) Finally, the detection of genomic RNA in the cytoplasm did not contradict published findings. In situ hybridization of the HDV RNA in HDV-infected hepatocytes (6) showed concentrated grains in the nucleus and also dispersed grains in the cytoplasm. The authors concluded that HDV RNA replication occurred mainly in the nucleus but overlooked the presence of the HDV RNA signals in the cytoplasm. Clearly, in light of the results reported in the present study, the presence of HDV RNA in the cytoplasm should not be ignored.

It is interesting that the characteristics of the HDV RNA species (monomer and dimer) in the nucleus and cytoplasm show significant differences. The cytoplasmic HDV RNA is usually more heterogeneous and often consists of multiple bands (Fig. 1, 4B, and 5). Furthermore, the dimer HDV RNA in the cytoplasm consists of only linear molecules, whereas that in the nucleus includes both linear and circular forms. Although we do not know the molecular mechanisms accounting for these differences, the possibilities that the nuclear and cytoplasmic HDV RNA may undergo different processing and that both nuclear and cytoplasmic species participate in HDV RNA replication will have significant implications for HDV replication. To investigate these possibilities, we attempted to examine the possible effect of blocking HDV RNA export on RNA replication by cotransfecting (with HDV RNA) an mRNA expressing vesicular stomatitis virus matrix protein (VSV-M), which is a potent nucleocytoplasmic transport inhibitor (26). We showed that HDV RNA replication was severely inhibited (data not shown). However, VSV-M also inhibited pol II-dependent transcription (but not pol I- or pol III-dependent transcription; data not shown); thus, we are uncertain at the present time whether this inhibition was the result of inhibition of nucleocytoplasmic transport or inhibition of pol II transcription (or both). Nevertheless, a possible cytoplasmic phase of HDV RNA replication remains a fascinating possibility.

The mechanism of preferential nuclear export of the genomic, but not antigenomic, HDV RNA is not clear. As monomer RNAs appeared to be the most efficiently exported and the multimer RNAs were not exported, ribozyme cleavage of HDV RNA may be closely linked to nuclear export. Since genomic HDV RNA was shown in an accompanying study (15)

to be synthesized by pol II, it is tempting to speculate that HDV RNA may be exported by the same mechanism as that for the splicing-dependent export of cellular mRNAs. In this regard, it is interesting that, similar to cellular mRNAs (reviewed in reference 4), HDV RNA is exported by a Crm1-independent pathway. This may also explain why antigenomic HDV RNA, which has been shown to be synthesized by an enzyme other than pol II (15), is not efficiently exported. Why antigenomic RNA is exported (although the total amount of RNA exported is small) early in HDV RNA replication cycle is not clear. It is conceivable that antigenomic HDV RNA is exported by a mechanism entirely different from that for genomic HDV RNA.

The preferential export of genomic HDV RNA may also have implications for the regulation of HDV RNA packaging into virion particles. Only genomic HDV RNA is packaged, and yet no specific RNA packaging signal has been identified (10; H. Meka, M. M. Lai, and T. B. Macnaughton, unpublished results). Moreover, since HBsAg is present only in the cytoplasm and HDV RNA replication occurs in the nucleus, the nuclear export of genomic HDV RNA at certain points of the viral replication cycle must also be required. It has been suggested that this is dependent on L-HDAg, which contains a nuclear export signal near the COOH terminus (11). However, the role of this signal in RNA packaging has yet to be demonstrated. The presence of genomic (but not antigenomic) HDV RNA in the cytoplasm offers an alternative mechanism for the selection of genomic, but not antigenomic, HDV RNA for packaging. In this study, a low level of antigenomic RNA export was observed very soon after RNA transfection. Since this event occurs prior to the expected appearance of L-HDAg, no packaging of this species would be expected under normal circumstances. Interestingly, in preliminary experiments using wild-type HDV cDNA transfection, we found that when L-HDAg (and HBsAg) is provided at the time of transfection, antigenomic wild-type HDV RNA can also be packaged (H. Meka et al., unpublished results), consistent with the possibility that the selectivity of HDV RNA packaging is determined by the availability of cytoplasmic HDV RNA rather than the presence of a specific packaging signal on the genomic RNA.

These findings have thus uncovered a previously unrecognized facet of HDV RNA replication, namely, genomic HDV RNA is exported and remains in the cytoplasm throughout most of the RNA replication cycle. Whether this cytoplasmic RNA merely participates in viral RNA packaging or is a necessary step of viral replication will be a fascinating issue. Clearly much further research will be required to determine the mechanism of export as well as the role(s) this event plays in HDV replication.

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